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Production of mannitol from inulin by simultaneous enzymatic saccharification and fermentation with *Lactobacillus intermedius* NRRL B-3693

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Abstract

The production of mannitol by *Lactobacillus intermedius* NRRL B-3693 using inulin as a substrate was investigated at pH 5.0 and 37 °C. The bacterium produced mannitol ($106.2 \pm 0.3\,g\,l^{-1}$) from dilute acid hydrolyzate (pH 2.0, 121 °C, 15 min) of inulin ($150\,g\,l^{-1}$) in 34 h. It also produced mannitol from inulin by simultaneous saccharification and fermentation (SSF) at pH 5.0 and 37 °C using inulinase ($8\,U\,g^{-1}$ substrate). From $300\,g\,l^{-1}$ inulin, the *L. intermedius* B-3693 produced 207.4 ± 1.2 g mannitol in 72 h by SSF. The fermentation time decreased from 72 to 62 h using a mixture of fructose and inulin (1:1, total, $300\,g\,l^{-1}$). When fructose and inulin mixture (3:5, total $400\,g\,l^{-1}$) was used as substrate, the bacterium produced 227.9 ± 1.8 g mannitol l^{-1} from both inulin and fructose with a yield of $0.57\,g\,g^{-1}$ substrate after 110 h of SSF. This is the highest level of mannitol ever produced by a microorganism reported in literature.
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Keywords: Inulin; Mannitol production; *Lactobacillus intermedius*; pH-controlled fermentation; Lactic acid production

1. Introduction

Mannitol, a naturally occurring polyol, is widely used in the food, pharmaceutical, medicine, and chemical industries [1]. It ($3.32\,lb^{-1}$; global market, 30 million lb $year^{-1}$) is currently produced industrially by high-pressure hydrogenation of fructose/glucose mixtures in an aqueous solution at high temperature (120–160 °C) with Raney nickel as catalyst [2]. Typically, the hydrogenation of a 50/50 fructose/glucose mixture results in an approximately 25/75 mixture of mannitol and sorbitol. Since the glucose is hydrogenated exclusively to sorbitol, this means that half of the fructose is converted to mannitol and half of it to sorbitol ($0.73\,lb^{-1}$). As a consequence, the commercial production of mannitol is always accompanied by the production of sorbitol thus resulting in an inefficient process [3]. Moreover, it is relatively difficult to separate sorbitol and mannitol, which results in even higher production costs and decreased yields [4].

Some microorganisms can specifically produce mannitol from glucose or fructose without making a sorbitol byproduct [1,5–7]. Mannitol at $180\,g\,l^{-1}$ can be easily recovered from the fermentation broth by cooling crystallization. Thus, research efforts have been directed towards production of mannitol by fermentation and enzymatic means [8]. Several heterofermentative lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Leuconostoc*, and *Oenococcus* have been reported to produce mannitol from fructose [3,9–12]. In our previous paper, we reported the production of mannitol and D-lactic acid by *Lactobacillus intermedius* NRRL B-3693 from fructose using a simplified MRS medium [13]. This (LAB) was selected after screening 72 bacterial cultures from the ARS Culture Collection, Peoria, IL. The method makes use of the capability of the bacterium to utilize fructose as an alternative electron acceptor, reducing it to mannitol with the enzyme mannitol dehydrogenase (EC 1.1.1.67) [14]. In this method, the reducing equivalents are generated by conversion of about one-third of the fructose to lactic acid and acetic acid [13].

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Inulin is a polyfructan, consisting of linear β-2,1-linked polyfructose chains terminated at the reducing end by a glucose residue [15]. This polymer is a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia. These inulin sources have recently received attention as a potential feedstock for fuel ethanol production [16]. Endoinulinases (2,1-β-D-fructan fructoanhydrolase, EC 3.2.1.7) are specific for inulin and hydrolyzes the internal β-2,1-fructofuranosidic linkages to yield inulotriose, inulotetraose, and inulopentaose as the main products. Exoinulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) split terminal fructose units from inulin. The synergistic action of endo- and exo-inulinases on inulin allows its efficient hydrolysis to fructose. In this paper, we describe the production of mannitol and lactic acid from inulin by *L. intermedius* NRRL B-3693 using a simultaneous saccharification and fermentation (SSF) approach.

2. Materials and methods

2.1. Materials

Inulin (extracted from the root of the chicory plant, *Cichorium intybus*, producer: Sensus, Roosendaal, The Netherlands) was purchased from Imperial Sensus, Sugarland, TX. Fructose syrup (Kryster liquid fructose), Soy peptone type D, and corn steep liquor were supplied by A. E. Staley Manufacturing Co., Decatur, IL; Marcor Development Corp., Carlstadt, NJ; and Cargill, Minneapolis, MN, respectively. Aminex HPX-87P and AminexHPX-87H columns (300 mm × 7.8 mm, each), and Carbo P and Cation H guard columns (30 mm × 4.6 mm, each) were purchased from Bio-Rad Laboratories, Hercules, CA. All other chemicals including inulinase from *Aspergillus niger* (Fructozyme L, Novozymes A/S, mixture of exo-inulinase, EC 3.2.1.80 and endo-inulinase, EC 3.2.1.7) were purchased from Sigma Chemical Co., St. Louis, MO.

2.2. Dilute acid hydrolysis of inulin

Inulin, at three different concentrations (150, 200, 250, and 300 g l^{-1}), was slurried in 18 mM H_2SO_4. The slurry was then adjusted to pH 2.0 with additional H_2SO_4 and treated in an autoclave at 121 °C for 15 min. The treated inulin hydrolyzate was adjusted to pH 5.0 with 10 M NaOH before use as a substrate for mannitol production.

2.3. Inulinase assay

Inulinase activity was assayed in a reaction mixture (0.5 ml) containing 1% (w/v) inulin, 50 mM acetate buffer, pH 5.0, and appropriately diluted enzyme solution. After incubation at 50 °C for 30 min, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid (DNS) method [17]. One unit (U) of inulinase activity is defined as the amount of enzyme which produces 1 μmole reducing sugar as fructose in the reaction mixture min^{-1} under the above specified conditions.

2.4. Bacterial strain

Stock cultures of *L. intermedius* NRRL B-3693 (obtained from the ARS Culture Collection, Peoria, IL) were maintained in 70% glycerol vials at −70 °C. It was transferred and maintained in agar (1.5%, w/v) slants made with simplified MRS medium [18] containing 10 g peptone, 5 g yeast extract, 2 g ammonium citrate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g disodium phosphate plus 10 g glucose as carbon source l^{-1} (final pH 6.5) at 4 °C. The simplified MRS medium described above (pH 6.5) was used for the preparation of seed culture. The medium and the substrate were sterilized separately at 121 °C for 15 min. A 250-ml Erlenmeyer flask containing 100 ml of the medium with fructose (20 g l^{-1}) was inoculated with a loopful of cells taken from a stock slant and incubated at 37 °C on a rotary shaker (130 rpm) for 24 h. This culture was used as seed culture.

2.5. Fermentation conditions

Batch culture experiments were performed in pH-controlled 500 ml fleakers with an initial medium volume of 300 ml at 37 °C as described previously [13]. The medium contained 5 g Soy peptone D, 50 g corn steep liquor, 2 g ammonium citrate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g disodium phosphate l^{-1} (final pH 5.0) plus substrate (acid hydrolyzed inulin, inulin, or inulin plus fructose syrup). The initial pH was set at 5.0 and it was maintained at the same pH level by adding 2 N NaOH. Cultures were stirred magnetically at 130 rpm using 1.5 in. stir-bars. Samples were withdrawn periodically to determine cell growth, sugar utilization, and product yields. For simultaneous saccharification and fermentation, inulinase enzyme (8 U/g inulin) was added to the fermentation broth at the time of inoculation.

2.6. Analytical methods

Cell growth was monitored by measuring optical density of the appropriately diluted culture broth at 660 nm. Sugar utilization and product analysis were performed using high pressure liquid chromatography (HPLC) (Spectra-Physics, San Jose, CA). For quantification of inulin, fructose and mannitol, an Aminex HPX-87P column was used. The column was maintained at 85 °C, and the compounds were eluted with deionized water (Milli-Q water, Millipore Corp., Bedford, MA) at a flow rate of 0.6 ml/min. For lactic acid and acetic acid analyses, an Aminex HPX-87H column was used. The column was maintained at 65 °C, and the organic acids were eluted with 5 mM H_2SO_4 or 10 mM HNO_3 at a flow rate of 0.6 ml min^{-1}. Peaks were detected by refractive index and identified and quantified by comparison to retention times of authentic standards.

3. Results and discussion

3.1. Production of mannitol from dilute acid hydrolyzate of inulin

HPLC analysis of the supplied commercial inulin preparation used showed that it contained 92.6% inulin, 2.8% fructose, 0.5% glucose, and 4.1% disaccharides. Acid hydrolyzates (pH 2.0,121 °C, 15 min) of inulin prepared from 150, 200, 250, and 300 g inulin l^{-1} were used as substrates for mannitol production

Table 1
Production of mannitol from dilute acid hydrolyzates of inulin by *Lactobacillus intermedius* NRRL B-3693 at 37 °C

Inulin concentration (%, w/v)	Time (h)	Maximum cell density ($A_{660\,nm}$)	Mannitol yield (g l^{-1})	Lactic acid yield (g l^{-1})	Acetic acid yield (g l^{-1})
15	34	7.4 ± 0.1	106.2 ± 0.3	23.3 ± 0.5	18.6 ± 0.1
20	84	8.1 ± 0.6	138.4 ± 6.1	29.9 ± 0.3	25.7 ± 0.0
25	110	6.4 ± 0.0	174.8 ± 1.1	35.9 ± 0.5	32.0 ± 0.4
30	43	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

The medium contained dilute acid hydrolyzates of inulin, soy peptone 5 g l^{-1}, and corn steep liquor, 50 g l^{-1}. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Values reported are averages from duplicate experiments.

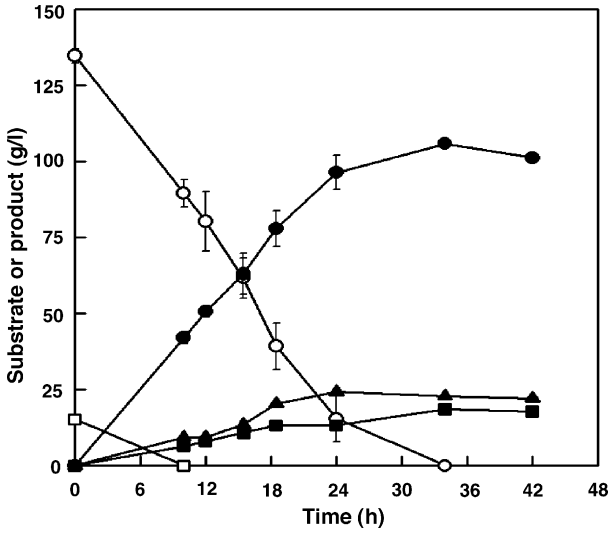

Fig. 1. Time course of substrate utilization and mannitol, lactic acid, and acetic acid production from inulin acid hydrolyzate at 37 °C in pH-controlled fermentation. The medium contained dilute acid hydrolyzates of inulin,150 g l^{-1}; soy peptone, 5 g l^{-1}; corn steep liquor, 50 g l^{-1}. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Values reported are averages from duplicate experiments. Symbols: (◯), fructose; (□), glucose; (●), mannitol; (▲), lactic acid; (■), acetic acid.

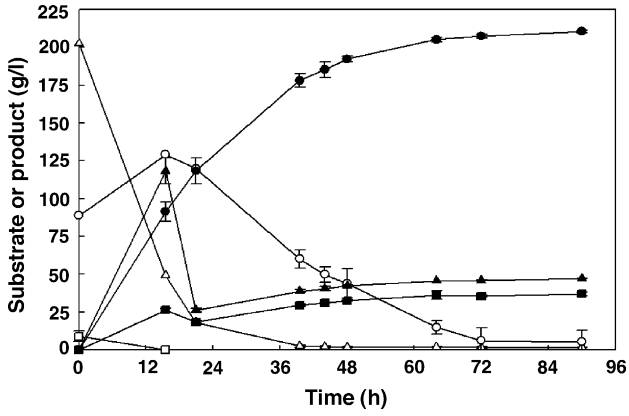

Fig. 2. Time course of simultaneous enzymatic saccharification and fermentation of inulin by *Lactobacillus intermedius* NRRL B-3693 at pH-controlled fermentation at 37 °C. The medium contained inulin, 300 g l^{-1}; soy peptone, 5 g l^{-1}; corn steep liquor, 50 g l^{-1}. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Inulinase (8 U g^{-1} substrate) was added at the time of inoculating the seed culture. Values reported are averages from duplicate experiments. Symbols: (△), inulin; (◯), fructose; (□), glucose; (●), mannitol; (▲), lactic acid; (■), acetic acid.

by *L. intermedius* B-3693. The results are presented in Table 1. It is evident that mannitol production was better at lower inulin concentration. With the increase of inulin concentration up to 250 g l^{-1}, the mannitol productivity decreased significantly even though the final mannitol yield was very good. At 300 g l^{-1}, the bacterium hardly grew and produced no mannitol tested up to 43 h. A time course of fructose utilization and products (mannitol, lactic acid and acetic acid) formation using inulin (150 g l^{-1}) acid hydrolyzate is shown in Fig. 1. The acid hydrolyzate did not contain any residual inulin which means that all inulin was hydrolyzed to mainly fructose and a little glucose.

3.2. Simultaneous enzymatic saccharification and fermentation of inulin by L. intermedius B-3693

The *L. intermedius* B-3693 strain did not possess any inulinase activity. Simultaneous enzymatic sacchrification of inulin by a commercially available inulinase preparation (8 U/g substrate) and production of mannitol by *L. intermedius* were performed at pH 5.0 and 37 °C using inulin (150–350 g l^{-1}) as substrate. The result is presented in Table 2. It is clear that SSF approach worked well for production of mannitol from inulin. Using inulin at 300 g l^{-1}, the bacterium produced 207.4 ± 1.2 g mannitol in 72 h (Fig. 2). The SSF approach offers some advantages over separate hydrolysis and fermentation (SHF): one pot conversion, time and energy savings etc.

3.3. Production of mannitol from two substrate system (fructose and inulin)

The production of mannitol by *L. intermedius* was investigated using two substrate systems (fructose and inulin) by SSF approach. The result is presented in Table 3. When fructose and inulin mixture (1:1, total, 300 g l^{-1}) was used as substrate, the bacterium produced 206.6 ± 0.8 g mannitol in 62 h (Fig. 3). These results indicate that the fermentation time decreased from 72 to 62 h by providing fructose along with inulin when compared to using only inulin as substrate. When fructose and inulin mixture (3:5, total 400 g l^{-1}) was used as substrate, the bacterium produced 227.9 ± 1.8 g mannitol l^{-1} with a mannitol yield of 0.57 g g^{-1} available substrate (Fig. 4). However, the substrates were not completely utilized even after 110 h of fermentation time. The fermentation broth still contained 10.4 ± 0.0 g inulin and 67.6 ± 3.5 g fructose l^{-1}. The reason is not clear.

Table 2
Production of mannitol from inulin by simultaneous saccharification and fermentation with *L. intermedius* NRRL B-3693 at 37 °C

Inulin concentration (%, w/v)	Time (h)	Mannitol yield (g l^{-1})	Lactic acid yield (g l^{-1})	Acetic acid yield (g l^{-1})
Inulin (15%)	34	99.8 ± 33.4	28.5 ± 3.5	16.3 ± 4.1
Inulin (20%)	42	138.8 ± 6.6	32.4 ± 0.2	25.1 ± 1.1
Inulin (25%)	48	174.1 ± 4.5	38.4 ± 1.8	30.5 ± 0.1
Inulin (30%)	72	207.4 ± 1.2	45.9 ± 0.3	35.5 ± 0.3
Inulin (35%)	90	222.0 ± 1.3	49.2 ± 0.0	37.8 ± 0.3

The medium contained inulin, soy peptone 5 g l^{-1}, and corn steep liquor, 50 g l^{-1}. Initial pH was 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Inulinase (8 U g^{-1} substrate) was added at the time of inoculating the seed culture. Values reported are averages from duplicate experiments.

Table 3
Production of mannitol from fructose and inulin by simultaneous saccharification and fermentation with *L. intermedius* NRRL B-3693 at 37 °C

Fructose–inulin concentration ($g\,l^{-1}$; ratio)	Time (h)	Mannitol yield ($g\,l^{-1}$)	Lactic Acid yield ($g\,l^{-1}$)	Acetic Acid yield ($g\,l^{-1}$)
250; 3:2	37	169.7 ± 2.5	37.5 ± 0.2	28.8 ± 0.5
300, 1:1	62	206.6 ± 0.8	42.1 ± 0.3	33.0 ± 0.1
400, 3:5	96	227.9 ± 1.8	46.1 ± 0.0	36.0 ± 0.4

The medium contained inulin, fructose, soy peptone $5\,g\,l^{-1}$, and corn steep liquor, $50\,g\,l^{-1}$. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Inulinase ($8\,U\,g^{-1}$ inulin) was added at the time of inoculating the seed culture. Values reported are averages from duplicate experiments.

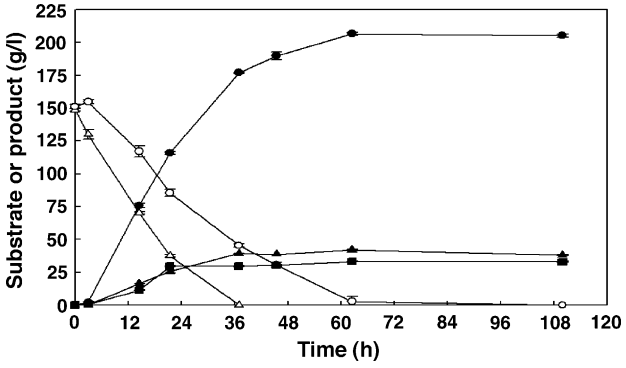

Fig. 3. Time course of mannitol, lactic acid and acetic acid production from fructose and inulin mixture (1:1, total, $300\,g\,l^{-1}$) in a pH-controlled fermentation at 37° C. The medium contained inulin, $150\,g\,l^{-1}$; fructose, $150\,g\,l^{-1}$; soy peptone, $5\,g\,l^{-1}$; corn steep liquor, $50\,g\,l^{-1}$. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Inulinase ($8\,U\,g^{-1}$ inulin) was added at the time of inoculating the seed culture. Values reported are averages from duplicate experiments. Symbols: (△), inulin; (◯), fructose; (●), mannitol; (▲), lactic acid; (■), acetic acid.

To my knowledge, this ($227.9 \pm 1.8\,g\,l^{-1}$) is the highest level of mannitol produced by fermentation by any microorganism reported in literature. In this respect, the bacterium is unique with its ability to tolerate very high concentrations of substrate and products. The mannitol production level is well above the cooling crystallization limit of mannitol which is $180\,g\,l^{-1}$. Thus, the bacterium can be easily used to produce mannitol at greater than $180\,g\,l^{-1}$. However, the bacterium grew poorly and produced little mannitol even at 144 h of incubation when a substrate concentration of $500\,g\,l^{-1}$ (fructose, $150\,g\,l^{-1}$ and inulin, $350\,g\,l^{-1}$) was used (data not shown). This indicates that this level of substrate inhibited the growth of the bacterium. Moreover, adding fructose to inulin (two substrate fermentation) does not provide additional benefit with respect to mannitol production.

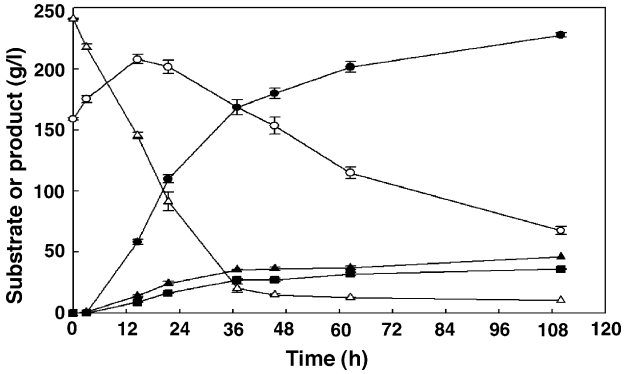

Fig. 4. Time course of mannitol, lactic acid, and acetic acid production from fructose and inulin mixture (3:5, total, $400\,g\,l^{-1}$) in a pH-controlled fermentation at 37° C. The medium contained inulin, $250\,g\,l^{-1}$; fructose, $150\,g\,l^{-1}$; soy peptone, $5\,g\,l^{-1}$; corn steep liquor, $50\,g\,l^{-1}$. Initial pH was at 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. Inulinase (8 U/g inulin) was added at the time of inoculating the seed culture. Values reported are averages from duplicate experiments. Symbols: (△), inulin; (◯), fructose; (●), mannitol; (▲), lactic acid; (■), acetic acid.

In conclusion, inulin containing substrates can be easily used for production of mannitol by fermentation where such renewable substrates are readily available. As mannitol is a much higher value product than ethanol and its demand is growing rapidly, it would be economically advantageous to make mannitol from inulin by fermentation.

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